MULTIPLEX IMMUNOASSAYS AS AN EFFECTIVE METHOD TO SIMULTANEOUSLY ANALYZE INFLAMMATORY MEDIATORS IN CENTRAL NERVOUS SYSTEM (CNS) CELLS: HUMAN ASTROCYTES STIMULATED WITH SARIN (GB).

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The central nervous system (CNS) is an immune-privileged site where the role of immune cells and mediators in brain injury caused by organophosphates (OP) is poorly understood. Many mediators have been identified in nervous system tissue. For instance, interleukin-6 (IL-6), a cytokine that acts on a wide range of tissue influencing cell growth and differentiation, is an agonist for vascular endothelial growth factor (VEGF). CNS cells producing IL-6 include astrocytes, macrophages, microglia, neurons, and brain endothelial cells. Here we describe the response of five different mediators, human interleukin-1beta (hIL-1β), hIL-6, hIL-8, tumor necrosis factor –alpha (hTNF-α), and human granulocyte macrophage-colony stimulating factor (hGM-CSF) associated with human astrocytes incubated with an OP, sarin (GB). Human astrocytes (~ 10^6 cell density) were stimulated with a high concentration of GB (0.8 mM) for 48 hours at 37° C. The expressed mediators in human astrocytes were detected by using the Luminex$^{100™}$ protein multiplex immunoassay. Constitutive non-stimulated human astrocytes secreted hGM-CSF (0.59 ± 0.03 ng/mL), hIL-1β (0.33 ± 0.05 ng/mL), hIL-6 (1.43 ± 0.02 ng/mL) and hIL-8 (0.39 ± 0.02 ng/mL). hTNF-α secretion was not detected or observed. GB decreased the endogenous secretion of these mediators as follows: hGM-CSF (0.51 ± 0.02 ng/mL), hIL-1β (0.28 ± 0.03 ng/mL), hIL-6 (1.00 ± 0.02 ng/mL). Meanwhile, the induction chemokine hIL-8 was increased 0.42 ± 0.03 ng/mL by GB as measured by Luminex$^{100™}$. Up and down-regulation of these mediators in human astrocytes promises a non-intrusive mechanism for assessment of the role of individual mediators in brain cell development, function and response to insult, such as that caused by the OP.

Many neurodegenerative disorders are associated with inflammatory processes in the central nervous system (CNS). The use of animal models to examine these phenomena and to develop therapeutic agents is frequently problematic, since regulation of many inflammatory mediators including hormones and cytokines in the CNS are fundamentally different between humans and rodents. Significant limitations exist in the application of rodent animal models in the evaluation of candidate anti-inflammatory or anti-viral therapies for human CNS diseases. We have developed isolation and primary culture systems for all major types of neural cells derived from human brains. Both neurons and astrocyte cells can be kept or grown in culture at high purity to allow a variety of cell biological approaches. Respecting the complexity of a human brain, it is our conviction that many fundamental processes in neural cells can be productively approached by first examining purified cells in culture, as is customary in many other fields of biomedical research. Cultured cells allow for experimental interventions that would be impossible to perform in vivo. These interventions include modulation of pro- or anti-inflammatory responses by defined compounds. Cultured neural cells are amenable to a variety of techniques to study functional genomics and proteomics.

Cytokine and chemokine signaling by astrocytes

Astrocytes play a key role in signaling networks in the brain that control development, growth, inflammatory processes and repair. Like astrocytes in vivo, cultured adult human astroyctes produce a wide range of cytokines, chemokines, and neurotrophic factors. Many molecular triggers influence this production. Thus, cultured astrocytes can be used to monitor the effects of candidate compounds that are designed to modulate signaling by cytokines, chemokines, or neurotrophic factors.

Cell culture and chemical treatments

Primary human astrocytes were obtained from Clonexpress Inc. (Gaithersburg, MD, USA). These cells did not differentiate into neuron. Human astrocytes were isolated from fetal brain tissue obtained from agencies authorized to procure and distribute such tissues for research. Brain tissue samples digested with collagenase and trypsin were plated in a proprietary medium that

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supports growth of both neuronal precursor cells and astrocytes. Following two weeks of growth in this medium, neuronal precursor cells were separated away from an enriched population of astrocytes. Cells were supplied as cryopreserved vials containing 2 x 10^6 viable cells. The cells were plated in tissue culture dishes. The second passage astrocytes were subcultured in 150 cm^2 flasks at a seeding density of ~ 2.5 x 10^3 cells per cm^2 in astrocyte growth medium for seven days.

Chemical treatments

Human astrocytes in 150 cm^2 culture flasks containing fresh media were exposed to different concentrations of the organophophorus nerve agent sarin (military designation: GB; isopropyl methylphosphonofluoridate) 0.80 to 0.22 mM per flask. Cell viability experiments (trypan blue exclusion) of controls (non-stimulated) and GB-stimulated cells showed that the cell viability for controls was greater than 95 % of surviving cells and approximately 85 % or lower with 0.8 mM GB under similar culture conditions. The culture flasks were maintained at room temperature in a chemical fume hood for approximately an hour and then transferred to a CO_2 incubator at 37 ^oC for 24 or 48 h.

Luminex$^{100™}$ Analysis System and Multiplex Antibody Reagent Kits

The general immunocytologic assay protocol is very similar to standard cytokine enzyme-linked immunosorbent assays. This novel method allowed the simultaneous measurement of 10 different biomarkers (i.e., human cytokines/ chemokine/ growth factors) and was designed to work in conjunction with the Luminex LabMAP™ system.

hIL-6 ELISA quantification

The standard cytokine ELISA was applied as previously described (Arroyo et al., 2003).

Summary

In the CNS, astrocytes are a major inducible source of interleukin 6 (IL-6). Furthermore, increasing evidence supports an essential role of IL-6 in the development, and differentiation as well as in de-and re-generation of neurons in the CNS. We show that IL-6 is released from constitutive non-stimulated human astrocytes. In addition, we show that stimulation of cultured astrocytes with GB decreases the secretion of hIL-6 as well as hIL-1β. We find that GB reduces hIL-6 in a dose-dependent manner. Our results indicate that IL-6 regulates specific expression in GB-stimulated astrocytes as shown by RT-PCR (Figure 1).

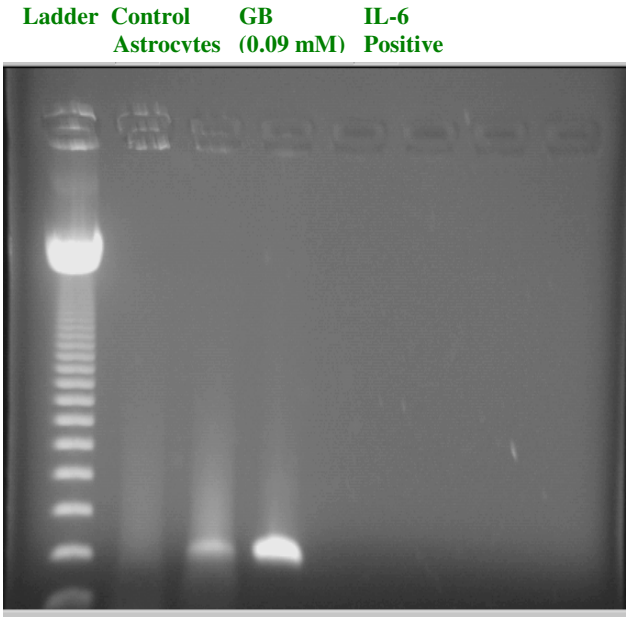

Figure 1. Differential expression of hIL-6 on control astrocytes and after stimulation with GB (0.09 mM).

IL-6 appears to have multiple effects, some neuroprotective and others damaging. The effects depend on the duration of exposure and the cell type (Hull et al., 1999; Orzylowska et al., 1999). Therefore, further studies are in progress to elucidate the role of IL-6 in diverse CNS functions such as protection of neuron from insults, neuronal survival, and neuro-immune responses caused by OP stimulation.

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